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14. ABSTRACT Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death in the United States. The most common DNA alteration associated with prostate cancer is hypermethylation in the regulatory region of certain genes, particularly in the promoter of the pi-class glutathione-S-transferase (GSTP1) gene. Analysis of hypermethylation of other gene promoters in combination has demonstrated high sensitivity and specificity for prostate cancer diagnosis. In this project, we evaluate the feasibility of detection of prostate cancer by molecular urinalysis. Prostatic manipulation from sources such as a biopsy needle, transrectal ultrasound (TRUS) probe, or digital rectal exam (DRE), may cause prostatic DNA to appear in the urine by shedding of neoplastic cells or debris into the prostatic ducts and urethra. The specific impact of prostatic manipulation on the detection of DNA promoter hypermethylation in the urine is unclear, as there are no studies comparing urine obtained before and after prostatic manipulation in identical patients. We hypothesized that voided urine specimens from patients with prostate cancer would be more likely to have detectable DNA promoter hypermethylation immediately after prostate manipulation by TRUS-guided needle biopsy than after DRE. We have compared voided urine samples obtained after extended (15-second) DRE with voided urine samples obtained after TRUS-guided needle prostate biopsy from patients with suspected or confirmed prostate cancer using conventional methylation-specific PCR (MSP) analysis to examine the hypermethylation status of three different gene promoters: GSTP1, APC and EDNRB. These loci were chosen because of their high frequency of methylation in prostate cancer specimens. Methylation analysis at multiple genes has also been shown to have diagnostic and prognostic value in prostate cancer.					
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INTRODUCTION:

Serum prostate-specific antigen (PSA) and digital rectal examination (DRE) remain the standard of care for prostate cancer screening despite their limited ability to detect occult prostate cancer. It is estimated that 15% of men with a normal PSA and DRE harbor prostate cancer. The rate of false negative prostate biopsies is estimated to be between 20-35%. Clearly, more specific and sensitive tests are needed to spare unnecessary biopsies and better identify and prognosticate affected men with prostate cancer. The scope of this research is to study, develop, and optimize biomarkers for the detection and prognostication of prostate cancer by molecular urinalysis. The biomarkers under investigation include AMACR (alpha-methylacyl-CoA racemase) [a protein], DD3/PCA3 (prostate cancer antigen-3) [an untranslated RNA], and GSTP1 (glutathione S-transferase pi) methylation [an epigenetic DNA marker] which may help discriminate benign from malignant conditions of the prostate.

BODY:

Specimen collection for biomarker analysis began since the funding started last summer. We have in addition tried to optimize methods of urine collection and storage. The molecular urinalyses in this project involve an assessment of DNA, RNA, and proteins in patient urine, and thus we have tried to standardize and optimize methodology for the collection of such molecules from clinical urine samples. Specifically, we have embarked upon the collection primarily of post-prostate biopsy (post-biopsy) urine, but also of post-digital rectal examination (post-DRE) urine. In terms of optimization of specimen handling, we have assessed the protein yield from clinical post-biopsy urine samples with an experiment where we collected post-prostate biopsy urine and immediately stabilized $\frac{1}{2}$ of the sample with a protease inhibitor tablet [CompleteTM (Roche Diagnostics)] and stored it at 4o C overnight, while the other $\frac{1}{2}$ was simply stored at 4o C overnight (a standard workable time between clinical collection and laboratory utilization). The next day these paired samples were prepared for total protein and then run on gels. Coomassie staining for total protein and Western blots for AMACR protein (with Ponceau S staining to verify protein transfer to nitrocellulose) were performed to assess whether the CompleteTM step was necessary or helpful in increasing protein yield. We found that the stabilization of urine samples with CompleteTM protease inhibitor pellets did in fact increase total protein yield, but unfortunately significantly increased the nonspecific background on our AMACR Westerns. Since even without protease inhibitors detectable AMACR in urine sediment was obtained, we now omit the addition of CompleteTM to our urine samples. We now simply store our urine samples at 4oC until the end of the day or early the next day, at which point they are spun down and separated into pellet and supernatant fractions. These are then stored at minus 80oC until ready for protein purification. To date, we have had success in preparing protein and detecting AMACR protein in the urine sediments (pellets), while our assessment of AMACR in urine supernatants has been below the level of detection by Western for the majority of analyzed samples, even in patients with large volume AMACR+ prostate cancers.

In terms of optimization of DNA yield from post-biopsy and post-DRE urine samples, we have developed good experience with the QiAmp viral RNA Mini kit (Qiagen, Valencia ,CA) for nucleic acid preparation from trace samples. Our experiments using unconcentrated urine supernatant did not yield PCRable DNA in a majority of cases, but when we used urinary sediment (pellet), we did get a PCRable DNA product from essentially all post-biopsy urine samples. Therefore, we have chosen to focus on the analysis of the post-biopsy urine sediment for our proposed DNA analyses (such as for methylation specific PCR of prostate cancer-specific methylation patterns).

Based on our mentors' experience with the assessment of DNA methylation in malignancies, and on our grant proposal, we focused first on post-prostate biopsy urine sediments and attempted to assess them for promoter hypermethylation of GSTP1. We broadened this to include an assessment of promoter hypermethylation of other prostate-cancer related genes also in an attempt to develop a

diagnostic panel. At the same time, we sought to compare the data from samples of urine collected post-biopsy from samples collected post-DRE. Our goal was to compare these modalities of prostate manipulation in terms of the yield of diagnostic nucleic acid material of prostatic origin that could be obtained following each. Similar assessments for protein yield are planned. For the methylation experiments, we assessed not only for GSTP1, but also for two other genes known to be frequently hypermethylated in prostate cancer (APC and EDNRB). Summarizing, our data suggest a high concordance between the ability to collect epigenetically modified DNA from patients either post-biopsy or post-DRE, though certain cases had methylation detected only post-DRE or only post-biopsy. Nevertheless, the concordance between data obtained with either modality was good enough to warrant continuing with either depending upon the clinical situation. Indeed, we have started to collect post-DRE urine on patients with prostate disease whether or not they will come to biopsy, in addition to post-biopsy voided urine samples. All urine samples are being stored not only for DNA preparation for hypermethylation analyses, but also for protein purification (for AMACR, other prostate-cancer specific proteins, and prostate-specific cytokines).

KEY RESEARCH ACCOMPLISHMENTS

- 1) standardized and optimized methodology for the collection of clinical urine samples after prostate biopsies
- 2) creation of a urine bank from patients with prostate diseases

The urine samples have been collected in a variety of ways following prostate manipulation to enrich them for prostate specific molecules (post-DRE, post-prostate biopsy), and processed and preserved for DNA and protein analysis. Our urine bank should provide an ideal medium in which to test biomarkers of varying specificities and sensitivities for cancer and other prostate disease detection and prognostication over time. The fact that we have good clinical follow-up on most patients (over 90% see us again) makes the urine bank most valuable. Our goal of developing and testing biomarkers in the urine for the development of a diagnostic and prognostic test for prostate cancer may be achievable if our urine bank grows large enough and if the urine continues to appear productive in terms of prostate-specific biomarkers. A test that would be more accurate than PSA and DRE is sorely needed.

REPORTABLE OUTCOMES:

Our preliminary results demonstrated that there is a high concordance between the ability to collect epigenetically modified DNA from patients either post-biopsy or post-DRE, though certain cases had methylation detected only post-DRE or only post-biopsy. This result has been submit as a manuscript for publication and is currently under review (Table 1 and Figure 1).

CONCLUSION:

Detection of prostate cancer by molecular urinalysis is feasible. We will continue to address our aims of collecting urine samples post-biopsy, and will assess them for biomarker information. In addition, we are planning to analyze the post-DRE and post-biopsy urine sediment (pellet) for intact prostate cells by cytoprep and immunohistochemistry now that prostate and prostate-cancer specific immunohistochemical markers are available. If we are able to detect prostate cells in the urine samples we collect, the next step will be to try and detect prostate cancer cells using relatively prostate cancer-specific immunohistochemical markers such as AMACR. The goal is to develop

another modality of urine analysis into a specific diagnostic test for prostate cancer, and to compare this test with the others we are studying in terms of accuracy of diagnosis and prognostic relevance if any.

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APPENDICES: N/A

SUPPORTING DATA:

Table 1. Clinicopathological characteristics and promoter hypermethylation detection status for GSTP1, APC, and EDNRB for 17 study patients.

No.	Age	PSA	DRE	Biopsy	clinical stage	Gleason	RRP stage	GSTP1	APC	EDNRB
218	65	8.8	-	-	N/A	N/A	N/A	U/U	U/U	NI/U
219	60	11.3	-	-	N/A	N/A	N/A	M/M	M/M	M/M
227	71	4.8	-	-	N/A	N/A	N/A	U/U	U/U	M/M
230	63	7.2	+	-	N/A	N/A	N/A	U/U	U/U	M/M
238	46	4.7	+	-	N/A	N/A	N/A	U/U	U/U	U/U
228	64	6.1	-	+	T1c	6	N/A	U/U	U/U	M/M
226	58	1.2	-	+	T1c	6	N/A	U/U	U/U	M/M
60	72	0.4	-	+	T1c	6	N/A	U/U	U/U	M/M
105	52	12	-	+	T1c	6	T2NxMx	U/U	U/U	U/M
176	59	4.4	-	+	T1c	6	T2cNxMx	U/U	U/U	NI/NI
213	59	5.9	+	+	T2b	4+3	T3aN0Mx	M/M	U/U	U/M
214	67	3.5	-	+	T1c	6	N/A	M/U	U/M	U/U
215	62	5	-	+	T1c	6	N/A	U/U	U/U	M/NI
216	72	9.6	-	+	T1c	6	N/A	M/M	M/M	NI/M
217	72	5.4	-	+	T1c	6	Radiation	U/U	U/U	NI/NI
225	68	4.9	-	+	T1c	6	T2N0Mx	U/U	U/U	NI/NI
232	69	7.8	-	+	T1c	6	N/A	U/U	U/U	M/M

Key: NI, noninformative; N/A, not applicable; M/M, post-DRE urine methylated/post-biopsy urine methylated; U/U, post-DRE urine unmethylated/post-biopsy urine unmethylated; M/U, post-DRE urine methylated/post-biopsy urine unmethylated; U/M, post-DRE urine unmethylated/post-biopsy urine methylated. Discordant urine pairs are bolded, other urine pairs are classified as concordant.

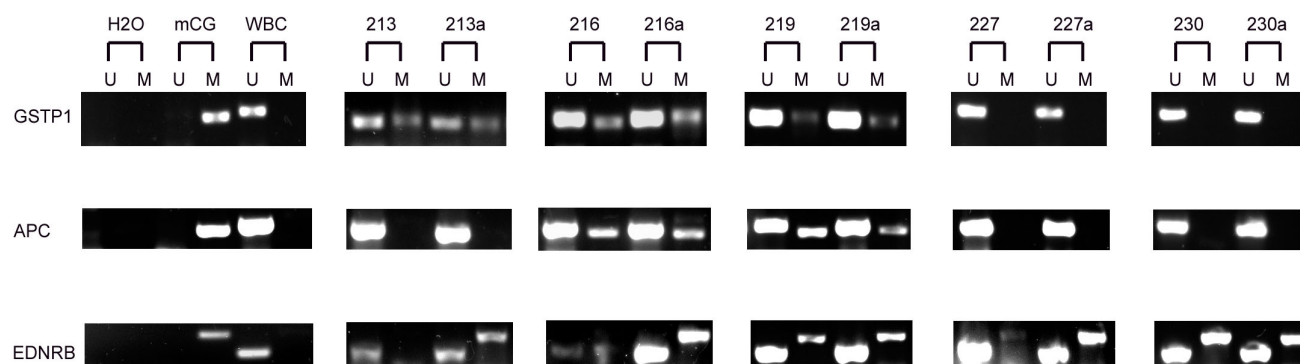


Figure 1: Representative Methylation-Specific PCR results for gene GSTP1, APC, and EDNRB. Paired urine specimens are ordered with post-DRE urine on the left and post-biopsy urine on the right (labeled "a"). Legend: *mCG*-universally methylated DNA (positive control); *WBC*-human male white blood cell DNA (negative control); *U*-primers specific for unmethylated CpGs; *M*-primers specific for methylated CpGs.